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14. ABSTRACT
During the reported period, our main goal was to test and further develop a hypothesis that structural modifications and/or interference between two heterochromatin proteins: MNEI and HP1 lead to abnormal gene regulation and impaired myeloid differentiation, CML acceleration, blast crises and/or secondary acute leukemia. In specific aim 1, we isolated monomeric and high molecular forms of MNEI by chromatography and gel electrophoresis and determined their primary structure by mass spectroscopy to identify the high molecular MNEI bands as those originating from MNEI and its complexes with a protease, neutrophil elastase. We designed peptides and raised antibodies against MNEI/elastase complex and specific peptides exposed in the modified MNEI. We found that these antibodies recognize MNEI65 expressed in a subset of CML cases including the blastic form of CML. In specific Aim 2 we conducted experiments on expressing MNEI and HP1 in leukemia cell lines. Cell cultures derived from a blastic CML cell model K562 expressing both MNEI and HP1 were raised and characterized for their chromatin structure and proliferation ability showing that expression of MNEI does not interfere with HP1 and other heterochromatin markers but significantly inhibits cell proliferation. This inhibition requires intact protease inhibitory activity of MNEI. Our results suggest that MNEI interferes with myeloid proliferation independently of heterochromatin proteins but through forming complexes with its target proteases such as neutrophil elastase.

15. SUBJECT TERMS
Chronic Myelogeneous leukemia/ Chromatin / Heterochromatin / MNEI protein / HP1 protein

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INTRODUCTION

Chronic myelogeneous leukemia (CML) initially develops through the chronic phase when the affected myeloid progenitors excessively proliferate but then undergo complete differentiation and become mature blood cells. When the myeloid progenitors respond poorly to the proliferation suppressing therapy, they eventually lose the ability to fully differentiate, and the chronic diseases transform into accelerated forms, blast crises, or acute secondary leukemia. The general goal of this work is to understand the molecular basis of the incomplete myeloid differentiation resulting from CML in order to improve the prognostics of the disease transition from chronic to accelerated forms and to design therapies capable of preventing or reversing the abnormal differentiation leading to the disease acceleration and death. To achieve this goal, we focused our study on two epigenetic heterochromatin factors associated with normal myeloid differentiation and CML: heterochromatin protein HP1 and MNEI. In terminally differentiated cells, HP1 is dramatically reduced but another heterochromatin-associated protein, MNEI, accumulates in chromatin of mature normal granulocytes. Our previous studies suggested that MNEI replaces HP1 to promote chromatin condensation and heterochromatin spreading in terminally differentiated myeloid cells. Based on two key observations that an altered high molecular isoform of MNEI accumulates in CML-derived cells and that MNEI and HP1 are co-expressed only in accelerated phase and blastic CML, we hypothesized that the structural modification of MNEI and its interference with HP1 leads to abnormal regulation of genes critical for myeloid differentiation and leading to partial cell de-differentiation, CML acceleration, blast crises and/or secondary acute leukemia.

BODY

During the reported period (17 months), the work involved the principal investigator (Dr. Sergei Grigoryev, 20% effort), the co-investigator (Dr. David Claxton, 2% effort), and the postdoctoral scholar (Dr. Evgenya Popova, 100% effort). The initial period of performance (1 March 2006 – 28 August 2007) was extended at no cost to 28 August 2008 with understanding that an annual report is due on 28 August 2007. The project was divided into two tasks (specific aims):

Task 1: To determine the primary structure of MNEI isoforms and their association with chromatin and HP1 in normal and CML-derived myeloid blood cells. In this set of experiments, we worked on isolating and identifying the primary structure of the high molecular forms of MNEI: MNEI^{72} and MNEI^{65}. Dr. Claxton provided cryopreserved blood samples where we
detected high level of MNEI\textsuperscript{65} using Western blotting. We also collected discard white blood cells from normal blood donors that contained high levels of MNEI\textsuperscript{72}. During the first stage of this work we had our IRB Protocol for work with discard normal blood and CML patients to be examined and found to comply with USAMRMC human subject protection regulations on 12 June 2006. We then isolated nuclei from human neutrophils and peripheral blood mononuclear cells (PBMC), produced nuclear extracts by treatment with 0.35 M NaCl and separated MNEI\textsuperscript{72} and MNEI\textsuperscript{65} using ion-exchange chromatography on FPLC system (Pharmacia).

Initially, the MNEI yield was insufficient for analysis but we overcame this obstacle by modifying the elution buffer and including appropriate dialysis before loading on the chromatography column. The column eluates were analyzed by Western blotting with anti-MNEI antibodies (Fig. 1) showing a very abundant band of monomeric MNEI\textsuperscript{42} (*) containing and higher molecular weight bands for MNEI\textsuperscript{72} (*** and MNEI\textsuperscript{65} (**). The fractionated proteins were separated on SDS-PAGE, bands of interest were excised, alkylated, and trypsin digested. Mass spectrometry was conducted in-house at the Penn State College of Medicine Macromolecular Core Facility equipped with the state of the art Applied Biosystems 4700 Proteomics Analyzer (MALDI TOF-TOF) with collaboration of Dr. Bruce Stanley, the facility director. We compared the molecular weights of peptides from the isolated protein with those theoretical trypsin-derived peptides of MNEI in databases using PeptIdent software and identified and mapped the peptides originating from the monomeric MNEI and its high molecular weight MNEI\textsuperscript{72}. These peptides appear to cover the beginning and the end of predicted MNEI sequence (Fig. 2) confirming that the complete MNEI molecules are present in the low- and the high molecular weight MNEI forms.

The yield, and especially the representation of MNEI\textsuperscript{65} peptides in mass-spectrometry was significantly lower than for MNEI\textsuperscript{72} and so far we were not able to unambiguously identify its binding partner from the masspec data. The molecular weight of one MNEI isoform (72 kDa) indicated that this band represents the complex of MNEI with its natural target in myeloid cells,
neutrophil elastase. Additional information that became available from the literature confirmed that MNEI forms an SDS-stable complex with neutrophil elastase (1) and that the MNEI-neutrophil elastase complex is translocated to the nucleus (2). We confirmed this hypothesis by forming MNEI-elastase complex that by its electrophoretic mobility is similar to MNEI$^{72}$. We concluded that MNEI-neutrophil elastase complex is formed in myeloid cells, and is likely to be found in the mature granulocytes of CML patients in the form MNEI$^{72}$. Further experiments showed that both MNEI$^{72}$ and MNEI$^{65}$ bands reacted with antibodies to MNEI-elastase complex.

After initial analysis of the high molecular forms of MNEI and positive identification of MNEI it was still unclear if the other high molecular weight form(s) could be attributed to RNA sequence alterations (alternative splicing, mutations) rather than posttranslational modifications or protein homo- and heterodimers. This was also an important to examine since a recent publication (4) has revealed MNEI mutations associated with other (non-leukemia) types of cancer. To address this possible sequence variations, we used RT-PCR to clone and sequence the cDNA and the potential mRNA splice variants of MNEI. Screening of normal blood and a number of leukemia samples including all samples expressing the MNEI$^{65}$ isoforms (3) showed no variations from the canonic human MNEI sequence. We thus can practically rule out the possibility that the high molecular weight forms of MNEI represent splice variants or mutant proteins. This is an important confirmation of our initial hypothesis that posttranslational protein modifications are responsible for MNEI alterations seen in CML blood.

After coming to the conclusion that DNA and RNA sequence variations are unlikely to cause the observed protein modifications, we used the massspectrometry data to build the maps of MNEI peptides. Importantly, the MNEI$^{72}$ map contains a peptide (RFQSLNADINKR) that is absent from the monomeric form. Because the monomeric MNEI peptides are much more abundant than MNEI$^{72}$ peptides, we concluded that this peptide is modified in the monomeric form. To test the expression of the MNEI isoforms we raised the following rat polyclonal antibodies: #28 against the whole MNEI-elastase complex; #29 against synthetic MNEI peptide RFQSLNADINKR; #30 against synthetic MNEI peptide KDKRTKVMMYQKKK exposed in

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Figure 2. MNEI protein sequence showing the peptides identified by mass spectrometry of MNEI$^{42}$ only (single underline), MNEI$^{72}$ and MNEI$^{42}$ (double underline) and MNEI$^{72}$ only (thick red line) as well as the peptide exposed in MNEI-neutrophil elastase complex (dashed blue line).
MNEI-elastase complex and targeting MNEI into the nucleus. The antibodies were raised commercially at Cocalico Biologicals, Inc (Reamstown, PA). We used Western blotting to test these antibodies against isolated MNEI and blood samples derived from various myeloid cells and CML patients (Fig 3). Our results show that all three antibodies recognized a subset of MNEI$^{65}$ and MNEI$^{72}$ isoforms expressed in a subset of CML cases including the blastic form of CML but also several chronic CML cases (Fig. 3). It thus appears that the high molecular form of MNEI represent a complex of MNEI with several proteins including MNEI-elastase which is variably expressed in different cases of CML. In the remaining work period, we will conduct immunofluorescence experiments to monitor the levels of MNEI-elastase complex across a large number of morphologically sorted nuclei from myeloid cells and leukemia samples stained with new antibodies in combination with HP1, histone H3 methylation, and total chromatin DNA in order to determine the extent of its correlation with particular CML and related MPD cases.

Figure 3 Western blotting of protein from the nuclei of peripheral blood mononuclear cells isolated from patients diagnosed with: CML, blastic form (bc); CML, accelerated phase (ac); CML, chronic phase (C); proliferative form of chronic myelomonocytic leukemia (CMML), unclassified myeloproliferative disorder (MPD); acute myeloid leukemia (AML). Nuclear samples were normalized to equal loading of histones before electrophoresis. The blots were probed with rabbit polyclonal antibodies against total MNEI (top panel) and rat polyclonal antibodies #28 against MNEI-neutrophil elastase complex, rat polyclonal antibodies #29 against MNEI peptide RFQSLNADINKR, and rat polyclonal antibodies #30 against MNEI peptide KKDRKTVKMMYQKKK (panels marked accordingly)
Task 2. To determine the chromosomal loci and genes directly affected by MNEI/HP1 interference.

We constructed MNEI-expressing and HP1-expressing vectors by subcloning the sequences human MNEI cDNA (Genbank Acc# M93056) and the MNEI14T->R mutant as well as HP1alpha (Genbank acc# AF21690_1) plasmids that we already have in the pEGFP-C3 expression vector and test the constructs by sequencing. We used this vector because it delivered a high level of protein expression without induction and the GFP protein conjugation highly improved the selection process for raising the cell lines and also served as an excellent control for the expression level with and without the protein fusion.

![Figure 4. K562 cells – control and transfected with HP1-GFP, MNEIwt-GFP, and MNEI14T->R-GFP visualized using differential interference (DIC) microscopy, and direct fluorescence with Hoechst 33258 (blue) showing DNA and GFP fluorescence (green) showing the localization of HP1 and MNEI as indicated. The control cell does not have green fluorescence.](image)

We transfected the wild-type MNEI and MNEI14T->R as well as the HP1 constructs into K562 and U937 cells and selected the transfected cells using neomycine resistance marker and then selected the clones expressing MNEI and HP1 as well as control vectors expressing GFP from the same promoter using both GFP fluorescence and immunofluorescence with specific antibodies. Both cell types produced a high level of expression with both types of genes seen as GFP fluorescence with nuclear localization for HP1 and nucleocytoplasmic for MNEI (Fig. 4). For K562 cells, we observed that HP1 and MNEI14T->R expression were not deleterious for the
cells over a long (more than several weeks) incubation period and we successfully established the HP1 and the MNEI-expressing clones using selection with neomycine. In contrast, with U937 in spite of testing several alternative transfection protocols (Lipofectamine, GenePorter, FuGENE) and achieving a very high level of initial transfection using electroporation with Cell Line Nucleofector kit V (Amaxa Inc.) method, we could not obtain cells that would survive while transfected with expression vector. The survival rate did not depend on the nature of the protein and we concluded that this type of cells was not amenable for stable transfection under our conditions. Because in the absence of either MNEI or HP1 expression, the vector alone was sufficient to eliminate the transfected cells from population especially under neomycin selection we concluded that even without protein induction the vectors were deleterious for these cells. We therefore focused our research on the work with K562 cell lines expressing MNEI and HP1.

![Figure 5. Top panels: results of FACS analysis of K562 cells expressing a) HP1-GFP, b) MNEI_{T>R}-GFP, and c) MNEI_{wt}-GFP after a period of 2.5 months selection with G-418. Upper right quadrants represent alive GFP-fluorescent cells. Panel d: percent of live cells population expressing HP1-GFP, MNEI_{T>R}-GFP, and MNEI_{wt}-GFP after a period of 6 days or 2.5 months selection with G-418.](image)

We then characterized the growth rates, chromatin condensation, and nuclear localization of MNEI and HP1 co-expressed in K562 as well as chromatin localization of endogenous methylated histone H3 by direct fluorescence of GFP using FACS sorting as well as by and immunofluorescence with antibodies against HP1 and H3me3(K9) and by Western blotting. We found that in these cells, MNEI expression did not interfere either with nuclear
localization of HP1 or with other heterochromatin markers. Expression of either HP1 or MNEI_{T->R} was not deleterious for K562 so that we could select and propagate cell cultures expressing these proteins for more than 2.5 months (Fig. 5 a,b). Expression of wild type MNEI while initially as efficient as with MNEI_{T->R}, upon prolonged incubation lead to a gradually decreasing number of living cells expressing MNEI_{wt} (Fig. 5 c,d). Having additional information that MNEI in complex with neutrophil elastase enters the cell nucleus and induces apoptosis (2) and that MNEI plus elastase produce the MNEI^{72} isoform, we are now conducting experiments aimed on coexpressing MNEI with neutrophil elastase and monitoring the apoptotic process in these cells. We have also successfully developed the conditions for chromatin immunoprecipitation (ChIP) and RT-PCR using real-time PCR detection with key genes associated with CML such as junB and c-myc as well as beta-globin as a control and we are conducting ChIP experiments aimed on identifying the genes altered by HP1 and MNEI coexpression. These experiments are currently in progress.

KEY RESEARCH ACCOMPLISHMENTS
- we isolated and conducted mass-spectrometric mapping of high molecular weight isoforms of MNEI associated with CML.
  - The high molecular form of MNEI was found to represent a complex of MNEI with several proteins including MNEI-elastase that is variably expressed in different cases of CML.
  - MNEI mRNA sequences were not altered in CML patients and it was concluded that the CML-linked changes are associated with posttranslational protein modifications.
- we established conditions for highly efficient and quantitative RT-PCR of MNEI and detected a peak of MNEI expression correlated with CML acceleration.
- MNEI expression was established in K562 cells and used to demonstrate that MNEI accumulation in the nucleus does not interfere with its putatively antagonistic protein HP1 in a short term but that expression of wild-type MNEI is significantly inhibited cell proliferation over prolonged incubation and this inhibition required intact protease inhibitory activity of MNEI.

REPORTABLE OUTCOMES
- a preliminary account of this work was reported at the “Road to a cure: the chronic myelogeneous leukemia research program meeting”, December 06, Orlando, Florida.
- Sequences of PCR primers and RT-PCR and real time PCR conditions optimized for quantitative MNEI analysis and CML acceleration diagnostics has been designed and tested.
- 11 vectors expressing high levels of HP1 and MNEI without deleterious cell effects have been constructed and tested.
- 3 cell cultures of K562 cells stably expressing MNEI<sub>wt</sub> (4 clones), MNEI<sub>T>R</sub> (4 clones), and HP1 (1 clone) have been constructed, selected, and propagated.

- 3 different rat polyclonal antibody preparations were raised and tested to specifically recognize high molecular weight MNEI isoforms.

CONCLUSION

In the reported period, we were able to confirm several important elements of our initial hypothesis, in particular we concluded that structural modifications rather than mutation or splice variants alter MNEI in myeloid cells and that variations in the level of MNEI<sup>65</sup> are associated with impaired myeloid differentiation and/or CML acceleration. We also prepared and validated new tools such as vectors, expressing cell lines, and antibodies against MNEI<sup>65</sup> that are necessary to conduct the final test of the hypothesis linking co-expression of MNEI and HP1 with impaired myeloid differentiation.

Importantly for biomedical applications, in particular for diagnostics and prognostics of CML, the information and the tools that we have obtained may help one to use expression of MNEI isoforms for identifying transitional stages in CML progression that serve as early indicators of CML acceleration where the course of CML treatment may be altered to accommodate to the changing phase of the disease.

REFERENCES


